# Surface-grafted antimicrobial drugs: Possible misinterpretation of mechanism of action

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Antimicrobial surface coatings that act through a contact-killing mechanism (not diffusive release) could offer many advantages to the design of medical device coatings that prevent microbial colonization and infections. However, as the authors show here, to prevent arriving at an incorrect conclusion about their mechanism of action, it is essential to employ thorough washing protocols validated by surface analytical data. Antimicrobial surface coatings were fabricated by covalently attaching polyene antifungal drugs to surface coatings. Thorough washing (often considered to be sufficient to remove noncovalently attached molecules) was used after immobilization and produced samples that showed a strong antifungal effect, with a log 6 reduction in *Candida albicans* colony forming units. However, when an additional washing step using surfactants and warmed solutions was used, more firmly adsorbed compounds were eluted from the surface as evidenced by XPS and ToF-SIMS, resulting in reduction and complete elimination of *in vitro* antifungal activity. Thus, polyene molecules covalently attached to surfaces appear not to have a contact-killing effect, probably because they fail to reach their membrane target. Without additional stringent washing and surface analysis, the initial favorable antimicrobial testing results could have been misinterpreted as evidencing activity of covalently grafted polyenes, while in reality activity arose from desorbing physisorbed molecules. To avoid unintentional confirmation bias, they suggest that binding and washing protocols be analytically verified by qualitative/quantitative instrumental methods, rather than relying on false assumptions of the rigors of washing/soaking protocols. *Published by the AVS.* https://doi.org/10.1116/1.5050043

## I. INTRODUCTION

Microbial biofilm infections on medical devices and implants continue to present challenges in modern health care. There are many reports on the proposed materials science solutions for combating pathogens or preventing their adherence onto medical devices.[1–5] One area of interest is surfaces with covalently attached antimicrobial agents.[6] These surfaces could prevent microbial colonization of biomaterials by an effective and persistent antimicrobial effect mediated by surface-contact. Such contact-killing surfaces could offer a potential long-term solution as medical device coatings, providing that the surface is not fouled or degraded by cellular metabolism. If true, antimicrobial surface coatings may be one way to repurpose potent antimicrobial compounds which may be too toxic for systemic administration. Finding new ways to combat microbial pathogens by repurposing drugs as surface coatings and reducing antibiotic use are ideas worth strongly considering in light of increasing antimicrobial resistance.

Preparing materials with covalently attached molecules is usually not synthetically challenging; however, the more important role played by surface adsorption and unintended release of weakly adsorbed molecules is a greatly underappreciated problem.[7] During the formation of a covalent bond between a surface and molecules in solution, surfaces are first activated with chemical functional groups and/or linking chemistries which make the formation of covalent bonds with solution molecules thermodynamically favorable. To ensure that reactions are driven to completion in a timely manner, it is typical to use a large excess of dissolved compounds, incubated with the activated surface over many hours. With excess molecules in solution, there is potential for much additional free energy gain through the formation of multiple weak interfacial bonds between a molecule and the surface, giving rise to (noncovalent) adsorption, which becomes superimposed on the intended covalent immobilization. Such physisorption can lead to multiple overlayers. In some cases, surface adsorption seems to be the dominant mechanism accounting for the majority of compounds present on freshly prepared and washed surfaces. Simple washing with aqueous buffers and extensive soaking is not sufficient for removing all traces of adsorbed layers.[8] When such materials are used in microbiological assays, it is easy to envision the pitfall resulting from an assumption (not analytically verified) that only covalently linked compounds remain.[9] That is, a small fraction of highly potent antimicrobial compounds become reversibly desorbed from the surface resulting in an observable (and desired) antimicrobial effect. For unaware researchers, this can support a confirmation

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bias where the mechanism of action is assigned erroneously to a contact-killing mechanism. Materials on which this assumption is based are poor candidates for clinical translation since their intended function can only be short-term and is unlikely to be validated under the scrutiny of rigorous clinical testing standards.

Our research is exploring novel materials' coatings that combat fungal biofilm formation on medical devices. This is a challenging and underappreciated area of research within the broader field of antimicrobial surface coatings.[2] Pfaller and Diekema reviewed the epidemiology of fungal infections related to invasive *Candida* spp. showing the substantial morbidity and mortality associated with diseases, particularly related to fungal biofilms on implanted medical devices.[10] Fungal pathogens cause clinically significant infections on a range of medical device surfaces[11] and are an important contributor to clinically significant poly-Kingdom (bacterial and fungal) biofilms.[12,13] Hence, further research needs to be done on how to combat fungal pathogens with a view toward developing hybrid surface coatings that can eliminate both prokaryotic and eukaryotic pathogens when polymicrobial biofilms are present.[14]

Polyenes are a class of potent antifungal drugs with activity against many fungal species and are listed on the WHO's list of essential medicines.[15] Polyenes (structures shown in Fig. 1) are known to interact with sterols in cell membranes, causing interference with the transport of molecules and ions across the membrane, resulting in cell death.[16] They can be delivered extracellularly by large liposomes or drug/polymer conjugates to the cell membrane.[17] When drug activity is mediated extracellularly and has a known effect in the fungal cell envelope, it is worth investigating opportunities to present antifungals covalently attached as surface coatings to act as a contact-killing surface to prevent biofilm formation.[18]

The main topic to be addressed in this report is whether covalently attached polyenes on surfaces are effective in the contact-mediated prevention of fungal cell adhesion and biofilm formation on surfaces. Previous reports have focused narrowly on amphotericin B (AmB) where conjugation to materials has mainly been demonstrated for soluble conjugates: polymer constructs for drug delivery such as gum arabic, carbon nanotubes, and others.[19–25] For materials' surfaces, hydrogel materials capable of releasing AmB have been investigated for drug release with a long-lasting antifungal effect.[26] As far as we are aware, there is only one report of the antifungal effects resulting from AmB conjugation to surfaces.[18] Paulo *et al.* reported on a multistep method where AmB was conjugated to dextran polymers. These were then linked to nanoparticles which were in turn physically immobilized on surfaces. Results showed that when exposed to $10^3$ colonies of *C. albicans* in a surface-contact assay, 78% inhibition was observed. They probed the specific issue of elution from the surface by testing the extracted supernatant that had been in contact with surfaces, observing some leaching which accounted for less than 20% of the activity.

In this report, we investigate surface-contact-killing antifungal coatings relevant to the wider polyene drug class. We provide a detailed study of the potential for surface adsorption followed by diffusive release using surface analytical instrumentation. We have used two surface assays to investigate the microbiological effect using $10^6$ colonies of *C. albicans*. Our results serve as a cautionary tale as they illustrate how easily it might be possible to infer incorrectly a contact-killing surface mechanism of action if proper washing and surface analysis studies are not performed.

## II. EXPERIMENT

### A. Materials and methods

Propionaldehyde, sodium borate, and phosphate-buffered saline (PBS) tablets were purchased from Sigma Aldrich, Australia. Caspofungin, sodium cyanoborohydride, AmB, Can, Nat, and Nys were supplied by Sapphire Bioscience. Sodium dodecyl sulfate (SDS; 90%) and dimethyl sulfoxide were supplied by Chem Supply, Australia. Silicon wafers were purchased from Gritek, China, and were cut to 1 $cm^2$ and cleaned before use. Thermanox coverslips (No. 1, 13 mm diameter) were purchased from ProSciTech, Australia. Plates of Sabouraud medium with antibiotics were purchased from Thermo Fisher Scientific Australia and used as per instructions. *C. albicans* strain ATCC 90028 was provided by the National Mycology Reference Centre (South Australian Pathology, Australia).

### B. Plasma polymerization

The deposition of a thin polymeric coating by gas plasma polymerization was performed using a custom-built plasma reactor[27] operated with a 13.56 MHz power generator and matching network. Substrates were first treated with air plasma (approximately $2 \times 10^{-1}$ Torr, 40 W, 2 min) on both sides to remove any hydrocarbon contamination. After evacuating again, the vapor pressure of propionaldehyde was adjusted to $2.0 \times 10^{-1}$ Torr and plasma was ignited at 40 W. After 1 min, the plasma generator was switched off and the chemical vapor was allowed to continue flowing over the substrate for an additional 1 min. Freshly deposited polymer layers on Thermanox coverslips and silicon wafers were used for conducting microbiology assays and surface characterization techniques, respectively.

### C. Surface analysis

#### 1. X-ray photoelectron spectroscopy

A Kratos Axis Ultra-delay-line detector X-ray photoelectron spectrometer equipped with a monochromatic Al Kα source was used to analyze surface chemical compositions of the coated samples. An internal flood gun was applied for neutralizing the charging effects. Survey and high resolution C 1s spectra were recorded at 120 and 20 eV pass energy, respectively. Data were quantified and processed by CASAXPS (ver. 2.3.16 Pre rel. 1.6, Casa Software Ltd) using Shirley baseline correction. All spectra were adjusted for charge

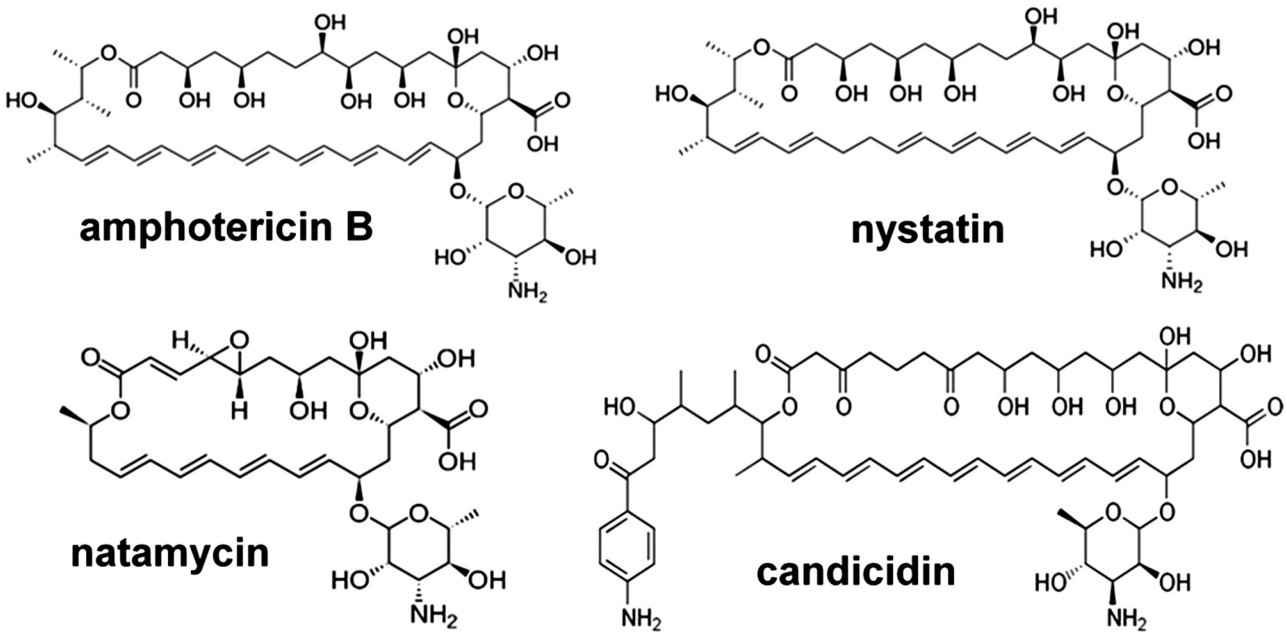


FIG. 1. Molecular structure of polyenes. In this paper, these are abbreviated as follows: amphotericin B (AmB), nystatin (Nys), natamycin (Nat), and candicidin (Can).

compensation effects by offsetting the binding energy relative to the C–C component of the C 1s spectrum, which was set to 285.0 eV.

### 2. Time of flight, secondary ion mass spectrometry

A PHI TRIFT V nanoToF instrument (PHI Electronics Ltd, USA) was used for time of flight, secondary ion mass spectrometry (ToF-SIMS) measurements. The system was operated at 30 kV energy under vacuum of $5 \times 10^{-6}$ Pa and armed with a pulsed primary $197Au^+$ ion beam to sputter and ionize species from each sample surface. PHI's dual beam charge neutralization system was used for charge neutralization. $CH_3^+$, $C_2H_5^+$, and $C_3H_7^+$ were used for positive mass axis calibration. Positive SIMS spectra were collected for a duration of 1 min from an area of 100 $\mu$m by 100 $\mu$m. Six data points were collected on each sample. The corresponding total primary ion dose was less than $1 \times 10^{12}$ ions cm$^{-2}$ and thus met the static SIMS regime.[28] A mass resolution $m/\Delta m$ of >7000 at nominal m/z = 27 amu ($C_2H_3^+$) was typically achieved. All recognizable, clear (i.e., unobscured by overlaps) fragment ions from 2 up to 100 amu range were used in calculations. The peaks were normalized to the total intensity of all selected peaks. Multiple mass spectra were processed with the aid of principal component analysis (PCA).[29] PCA was applied using NESAC/BIO Toolbox version v 2.8 along with MATLAB r2017a software version 9.2.

### 3. Spectroscopic ellipsometry

A variable-angle spectroscopic ellipsometer (J.A. Woollam Co. Inc., NE, USA) was used to determine the thickness of the polymer layer treated on silicon wafer samples. Data were attained with light wavelength scanned from 250 to 1100 nm in 10 nm intervals at 65°, 70°, and 75°. The optical properties of the silicon substrate were taken from the materials library in the modeling software (WVASE 32). The film thickness was estimated and refined by varying Cauchy parameters to achieve the best fit to the experimental data across the visible spectral range. Errors were estimated from the uncertainty associated with fitting.

## D. Binding and washing protocols

Plasma polymer surfaces were incubated for 30 min with 0.1 mg/ml polyene solutions in borate buffer (pH = 11) to assess binding polyenes onto aldehyde plasma polymer (ALDpp) in different buffer solutions. After this, an equal volume of 0.02 mg/ml $NaCNBH_3$ solution (reducing agent) in PBS was added and left for at least 5 h. Solutions were then extracted before washing the surfaces. Different washing protocols were applied separately in under different conditions to study the extent of removal of physisorbed compounds from the surface. In condition one, samples were washed with PBS buffer and purified water five times and three times, respectively. In condition two, samples were washed once with PBS buffer, and then, 0.2 M SDS solution was added to the substrates and incubated at room temperature for 15 min. After discarding this solution, samples were washed five times with PBS and three times with purified water at room temperature. In condition three, samples were washed once with PBS buffer. Then, prewarmed 0.2 M SDS solution was added to substrates and incubated at 70 °C for 15 min. This solution was aspirated and substrates were washed at room temperature five times with PBS buffer and three times with purified water.

### E. Microbiological evaluation of antifungal surface coatings

#### 1. Modified ISO22196 assay (contact assay)

A modified version of the ISO22196 protocol "Measurement of antibacterial activity on plastics surfaces" was adapted for use with yeast and used to count the number of colony forming units (CFU) per square centimeter of polyene-coated surfaces.[8] This assay is highly surface sensitive as a small volume of organisms is maintained in close contact with the sample surface and a sterile coverslip. On day 1, a couple of colonies were picked and suspended in 0.9% sterile saline solution and diluted to 0.5 McFarland units and then further diluted in Roswell Park Memorial Institute (RPMI) medium at 1:10 to generate a working concentration of $1 \times 10^5$. RPMI medium was obtained from Thermo Fisher Scientific Australia. Then, 75 $\mu$l of *Candida* suspension was added slowly to the center of each treated or control surfaces and then covered by an 18 mm diameter sterile glass coverslips and placed in a 37 °C incubator for 24 h. On day 2, each well was washed twice with 1 ml of RPMI medium containing 1% v/v Triton X-100 to remove adherent cells from surfaces. The washing solution from each well was pipetted into individual sterile 10 ml tubes and then diluted and spread on SAB-ANTI plates at 37 °C for 24 h. On day 3, the plates were counted and calculated as the number of colony forming units per square centimeter (relative to the area of the covered surface). For each condition, errors were estimated using three technical replicates.

#### 2. Static biofilm assay

A modified version of static biofilm assay was used to quantify the number of colony forming units per square centimeter of polyene-treated surfaces. Polyene coatings and control samples were placed in a 24 well plate. On day 1, 1 ml of *Candida* suspension at a concentration of $1 \times 10^6$ CFU/ml in spider medium was added to each well. 1 l of spider medium contains distilled water, 20 g nutrient broth, 20 g mannitol, 4 g $K_2HPO_4$, and pH was adjusted to 7.2 with NaOH. Then, the samples were incubated at 37 °C with shaking (80 rpm) for 90 min to allow adherence. After incubation, the contents from each well were aspirated and each sample was washed gently three times by adding 1 ml of PBS to remove nonadherent cells, and then the wash solutions were discarded. Subsequently, 1 ml of spider medium was added to each well and the plate was incubated at 37 °C for 24 h with shaking. On day 2, substrates were gently washed with 1 ml PBS to remove nonadherent colonies form surface. Then, each substrate was transferred to a 50 ml conical tube containing 10 ml sterile PBS and subjected to three cycles of vortex mixing for 1 min, followed by 1 min of sonication at 43 kHz. Then, the solution was transferred into a microcentrifuge tube and underwent a tenfold serial dilution. Dilutions (100 $\mu$l) were plated in duplicates onto SAB-ANTI plates and then incubated for 24 h at 37 °C. On day 3, colonies on the plates were counted. The number of colony forming units per square centimeter (relative to the area of the covered surface) was calculated.[30] For each condition, errors were estimated using three technical replicates.

## III. RESULTS AND DISCUSSION

The purpose of this work was to investigate whether polyene compounds can exert an antifungal effect when covalently immobilized as two-dimensional surface coatings. The most important aspects of this work were to demonstrate that (1) polyenes can be covalently surface immobilized and analytically verified after various washing protocols and (2) any observed antifungal effects were due to the surface-tethered compounds and not due to adsorbed molecules that were released from the surface.

### A. Polyenes can be covalently bound to surfaces

The intended covalent immobilization reaction is depicted in Fig. 2; it comprises well-known reductive amination.[31] The surface was functionalized with aldehyde groups via deposition of a thin plasma polymer derived from propionaldehyde vapor.[32] Polyene molecules, bearing a primary amine group, were grafted onto the polymer surface via imide bond formation in borate buffer at pH 11. Subsequently, these were converted to amine bonds by reduction with cyanoborohydride.

### B. XPS indicated the presence of bound polyenes

Chemical surface analysis using X-ray photoelectron spectroscopy (XPS) was used to indicate the presence of polyenes on surfaces. The ALDpp consisted of carbon, oxygen, and hydrogen atoms only, whereas surfaces grafted with polyene compounds have these atoms plus a small amount of nitrogen in their structures. XPS analysis showed that the ALDpp was composed of $90 \pm 0.5\%$ C and $10 \pm 0.5\%$ O. Table I shows that the surfaces, after grafting of polyenes, contained 1% or less of nitrogen. Detected amounts were low and approached the limit of detection of the instrument; however, values were not surprising given that there are only 1 or 2 nitrogen atoms present for every 65 or so carbon and oxygen atoms in the polyene structure. The N signal is further "diluted" against a background of ALDpp since the XPS sampling depth is around 10 nm, which substantially exceeds the thickness expected for a grafted monolayer of compounds of these molecular weights.

Samples were then washed using different chemical conditions and temperature. This led to decreases in the intensity of the N signal, indicating that the washing procedures removed physisorbed molecules, and to different extents. It is clear that washes using SDS or SDS at 70 °C reduced the amount of nitrogen on surfaces to likely monolayer coverage, with 0.2% N representing the limit of reliable detection with this XPS instrument. It is also evident that washing with PBS is not sufficient to remove physisorbed polyene compounds. Note that sulfur could not be detected indicating the absence of SDS on surfaces.

Given that N values were at or just above the limit of detection, and statistical differences were not significant,

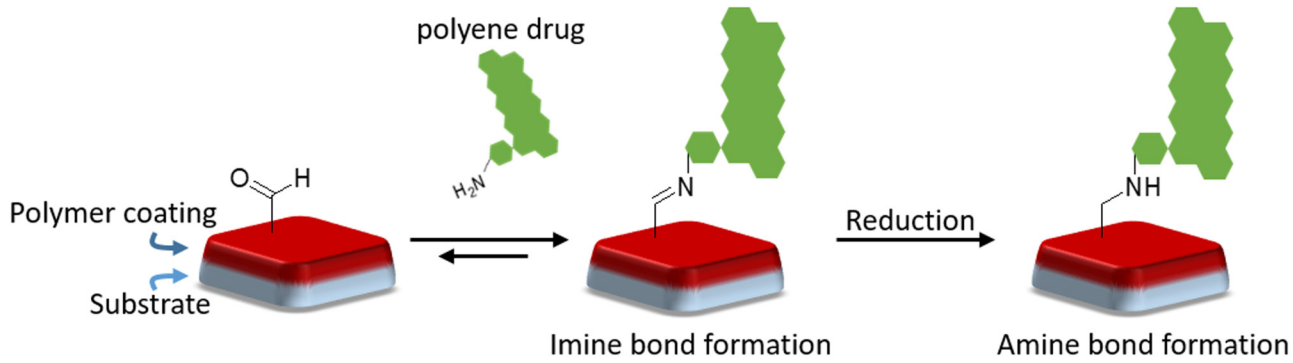


FIG. 2. Formation of covalent bonds between polyenes possessing a primary amine group and surface polymer coatings possessing aldehyde groups.

XPS cannot be exclusively relied upon to confirm the presence of polyenes. These data suggested the presence of polyenes and showed that washing influenced their release. It also confirmed that S was not present, showing that SDS was not adsorbed onto the surface. Further surface characterization was carried out using ToF-SIMS to confirm the presence of polyene molecules and changing amounts upon washing.

### C. ToF-SIMS confirmed molecular fragments from bound polyenes after SDS washing

ToF-SIMS was used to assess the presence of covalently grafted AmB on surfaces after the most rigorous washing condition (SDS at 70 °C). PCA was applied to assist analyzing and interpreting the complex ToF-SIMS data.[34,35] The difference between positive mass spectra recorded for the control ALDpp and the AmB-grafted sample was demonstrated by the scores plot shown in Fig. 3. The scores plot on principal component axes PC1 and PC2 shows that the positive mass spectra form two well-separated clusters, indicating differences between the surface chemistry of ALDpp and ALDpp-AmB samples. Figure 4 shows a representative loading of positive mass spectra for the ALDpp and ALDpp-AmB pair. The $C_2H_3O^+$ and $C_3H_3O^+$ fragments are key characteristics of ALDpp, whereas all the CH–$N^+$ and CH–$NO^+$ ions are associated with ALDpp-AmB. The principal positive loading on PC1 is $C_6H_8N^+$, $C_5H_{12}N^+$, and $C_4H_4NO^+$ fragment ions, which can be identified in the AmB structure (Fig. 4). While rigorous washing with SDS at 70 °C appears to have removed physisorbed AmB from the surface, analytical evidence shows that AmB remains on the surface, implying covalent bonding to the polymer surface coating.

TABLE I. Relative atomic percentages of elements from XPS survey data for polyene-grafted samples after different washing conditions. Uncertainties were estimated based on the analysis by Gengenbach *et al.* and cited papers within (Ref. 33). ND = none detected.

| Sample | Drug | C 1s (%) | O 1s (%) | N 1s (%) | S 2p (%) |
|---|---|---|---|---|---|
| ALDpp (reference) | none | 90.0 ± 0.5 | 10.0 ± 0.5 | ND | ND |
| Drug conjugated and washed with PBS | AmB | 81.3 ± 0.5 | 17.9 ± 0.5 | 0.5 ± 0.2 | ND |
| | Can | 82.5 ± 0.5 | 16.6 ± 0.5 | 1.0 ± 0.2 | ND |
| | Nat | 85.7 ± 0.5 | 13.7 ± 0.5 | 0.5 ± 0.2 | ND |
| | Nys | 84.5 ± 0.5 | 14.6 ± 0.5 | 1.0 ± 0.2 | ND |
| Drug conjugated and washed with SDS at 20 °C | AmB | 86.3 ± 0.5 | 13.1 ± 0.5 | <0.2 | ND |
| | Can | 85.0 ± 0.5 | 14.5 ± 0.5 | 0.6 ± 0.2 | ND |
| | Nat | 84.4 ± 0.5 | 14.9 ± 0.5 | 0.4 ± 0.2 | ND |
| | Nys | 85.5 ± 0.5 | 13.8 ± 0.5 | 0.6 ± 0.2 | ND |
| Drug conjugated and washed with SDS at 70 °C | AmB | 86.1 ± 0.5 | 13.4 ± 0.5 | <0.2 | ND |
| | Can | 85.7 ± 0.5 | 13.6 ± 0.5 | 0.2 ± 0.2 | ND |
| | Nat | 86.4 ± 0.5 | 13.2 ± 0.5 | <0.2 | ND |
| | Nys | 85.7 ± 0.5 | 13.5 ± 0.5 | 0.4 ± 0.2 | ND |

### D. Washing procedures did not damage the surface coating

Spectroscopic ellipsometry was used to measure the thickness of samples after binding and washing (Table II). The small thickness differences observed can be attributed to the removal of a small amount of low molecular weight polymeric material from the plasma polymer layer. While a thickness increase was observed after AmB binding, this change was relatively small and not significant enough to

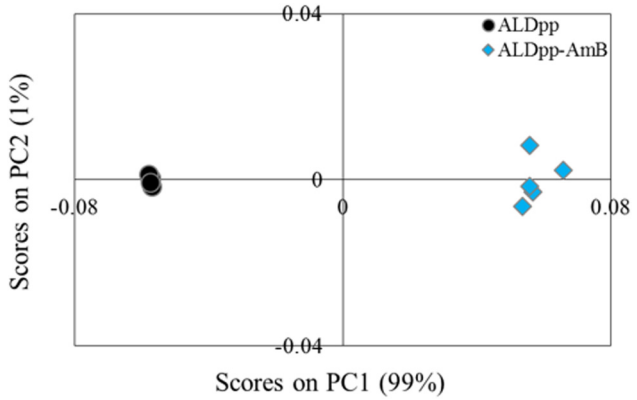


FIG. 3. Scores plot on PC1 and PC2 of positive mass spectra for ALDpp control and AmB surfaces. AmB was immobilized to ALDpp with reducing agent and then washed with SDS (70 °C) and finally PBS and water washes.

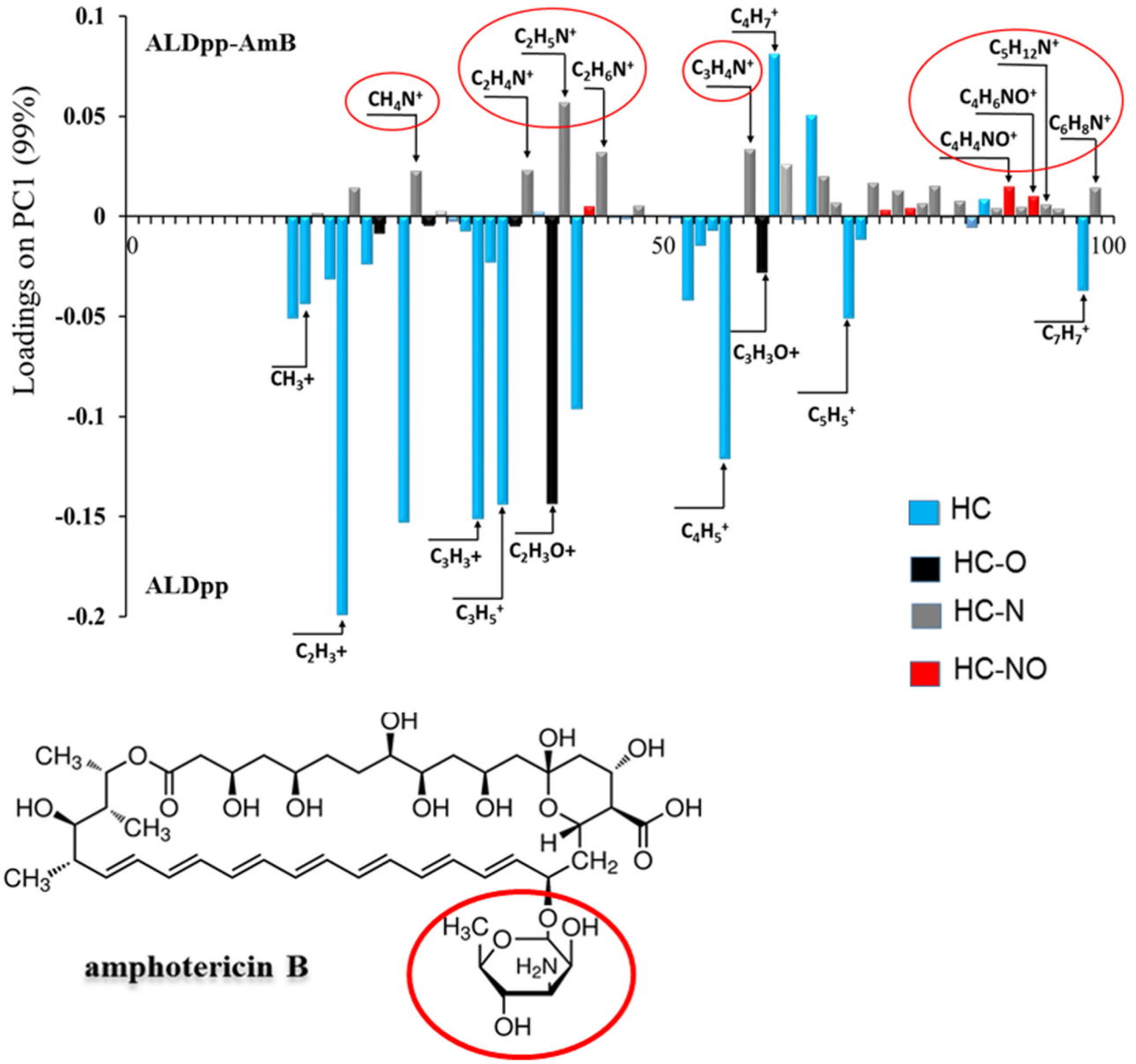


FIG. 4. Loadings on PC1 for positive mass spectra ion fragments for the AmB linked ALDpp surface, and the part of the AmB molecule that gives rise to the fragment ions showing up in the loadings plot.

confirm the presence of the compound, a point which was much better made using chemical characterization above. The point we wish to illustrate is that the robust washing conditions using heated surfactant solutions did not result in extensive damage or gross delamination of the plasma polymer coating.

TABLE II. Thickness measurements by ellipsometry of fresh ALDpp, after conjugating AmB and applying various washing treatments.

| Coating | Thickness (nm) |
|---|---|
| ALDpp (fresh) | 19.1 ± 0.1 |
| ALDpp + AmB + washed by PBS | 21.7 ± 0.1 |
| Above + washed by SDS at 20 °C | 21.1 ± 0.2 |
| Above + washed by SDS at 70 °C | 19.3 ± 0.1 |

### E. Antifungal activity with contact-killing and static biofilm assays

Two different microbiology assays were applied to investigate how fungal yeasts interact with the surface coatings. The contact assay evaluated the number of viable colony forming units surviving after being sandwiched between the coated surface and a sterile cover material. The small volume of inoculum used between the materials (75 $\mu$l) ensured that organisms were always within close proximity to the treated surface coating.

Figure 5 shows antifungal testing results using the contact assay for samples washed using different washing conditions.

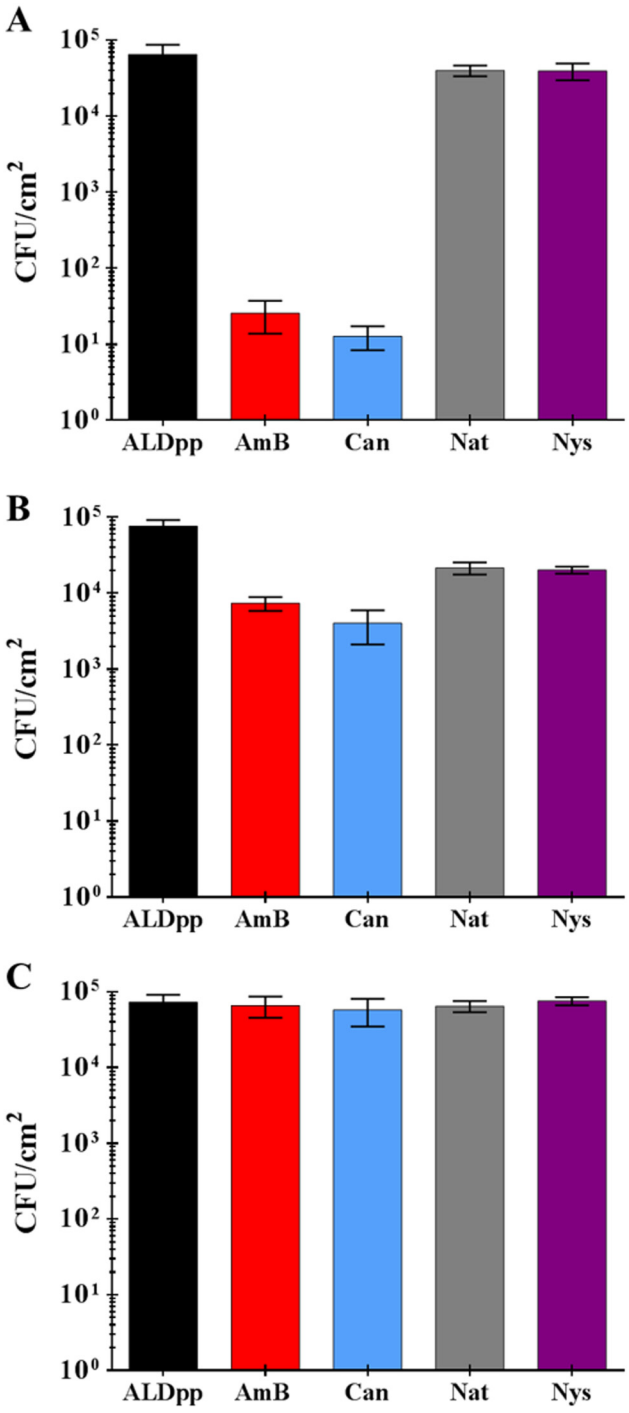


FIG. 5. Antifungal activity of surface coatings as determined by the contact assay. *C. albicans* colony forming units (mean and SD) on polyene-treated surfaces and controls washed with (a) PBS, (b) SDS 20 °C, and (c) SDS 70 °C.

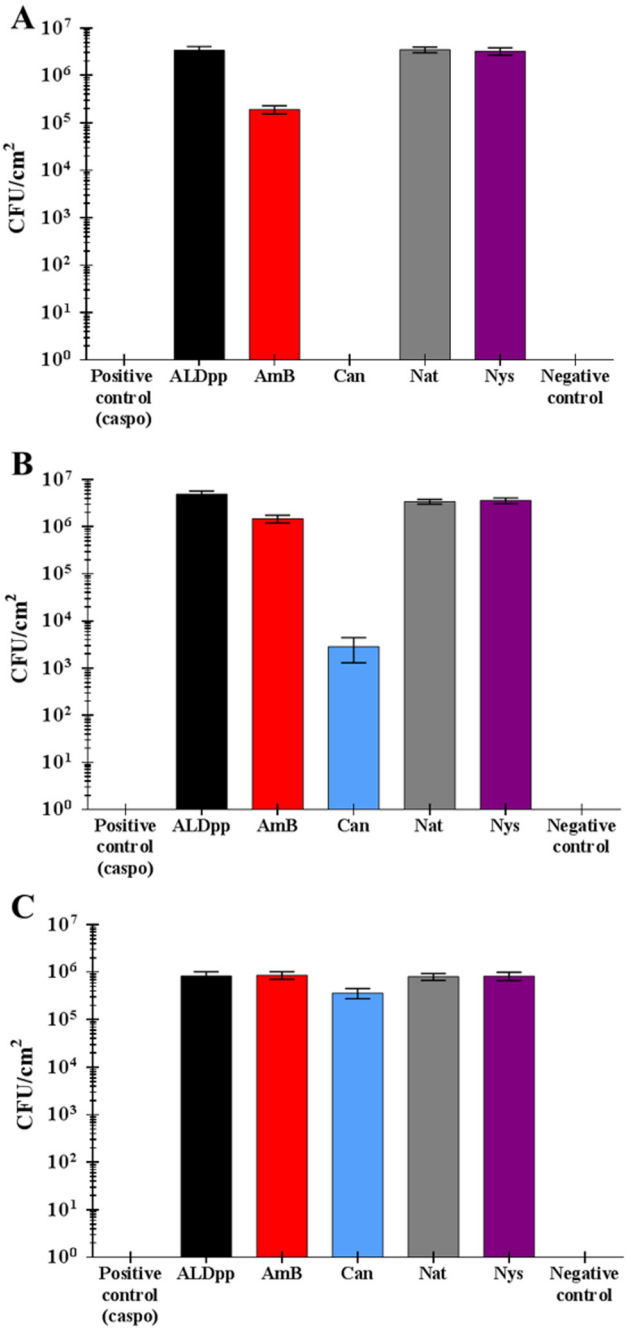


FIG. 6. Antifungal activity of surface coatings as determined by the static biofilm assay. *C. albicans* colony forming units (mean and SD) on polyene-treated surfaces and controls washed with (a) PBS, (b) SDS 20 °C, and (c) SDS 70 °C.

For samples washed with PBS, the results show a >log 3 reduction in viable colonies after exposure to AmB and Can surfaces. For Nat and Nys, almost no antifungal activity was observed. When more rigorous washing was used (SDS and elevated temperatures), the antifungal effect from AmB and Can was eliminated (i.e., indistinguishable from the control).

These results can be explained from the surface analysis data. XPS data indicated that PBS-washed samples bore polyene molecules that were adsorbed onto the materials by reversible bonding such as van der Waals forces. Thus, during the contact assay, these could elute from the surface coating, diffusing into solution and killing yeast cells. However, after rigorous washing, no antifungal effect was observed despite the presence of the compounds being analytically verified by surface analysis. No antifungal effect was observed for surfaces with Nat and Nys even after gentle washing conditions. This might be due to the fact that the small amounts eluted from washed surfaces failed to reach

the minimum inhibitory concentration (MIC) for *C. albicans*, compared to the much more potent AmB at the same concentrations.[36] The solution MIC for AmB is 0.12 $\mu$g/ml,[37] while for Nat, Nys, and Can it is 5, 3, and 0.5 $\mu$g/ml, respectively.[36,38] Additionally, we verified that in solution the MIC for AmB remained unchanged when solutions were heated to 70 °C for 15 min, suggesting that heating polyenes during washing could not explain the absence of antifungal activity on these surfaces. Thus, our observations support the conclusion that polyenes, when conjugated to surfaces through covalent bonds, are unable to cause an antifungal effect.

This proposition was further tested with a different antifungal surface assay. The static biofilm assay differs from the contact assay by interrogating the initial attachment phase of yeast cells to surfaces, and their subsequent ability to form a biofilm. Here, samples are exposed to fungal inoculum for 90 min with shaking. When the inoculum is removed, adherent cells, if present, are then allowed to grow and form the biofilm when incubated in media. Figure 6 shows data for the static biofilm assay for polyene-conjugated surfaces washed using different protocols. Again, on PBS-washed samples, a large to moderate antifungal effect was seen for Can and AmB surfaces, respectively, showing the absence of the biofilm or a significant reduction for these surfaces. Washing with SDS reduced their efficacy and the antifungal effect was eliminated using elevated temperature washing. Results were slightly different between the two assays, most notable in results when PBS washing was used. However, the overall conclusion is the same: surface coatings with covalently attached polyenes are not antifungal. It should be noted that we have used as a positive control ALDpp surfaces conjugated with the echinocandin drug caspofungin. Not only does this sample serve as a positive control to show that the assay is working properly, but it also provides a comparison of two surface coatings with covalently attached antifungal drugs. With caspofungin-linked surfaces, despite being immobilized covalently onto the surface, it is clear that the drug is still able to meet its target and has an inhibitory effect, as we have previously demonstrated.[8,39]

### F. Discussion: The role of materials in understanding the mechanism of action of surface-attached antimicrobials

Washing studies and detailed surface analysis are essential in order to avoid erroneous conclusions about the mechanism of action of surface-conjugated antimicrobial agents. It is easy to establish a false hypothesis that can be seemingly confirmed when the potential contributions of surface-adsorbed antimicrobial agents are not taken into consideration.

This can readily be seen in this study where a significant antifungal effect was obtained, despite the surfaces being extensively washed with buffers after preparation [Figs. 5(a) and 6(a)]. Only when more rigorous washing was used, did the antifungal effect abate and nullify under washing conditions of SDS used at 70 °C [Figs. 5(b), 5(c), 6(b), and 6(c)]. The use of several complementary surface analytical techniques was essential to confirm the intended attachment.[40] Surface analysis by ellipsometry, XPS, and ToF-SIMS confirmed the presence of the polymer layer with attached antifungals after this washing.

The reason why surfaces with covalently attached polyene molecules are not antifungal may be understood by the known mechanism of action for these drugs. Polyenes in solution are believed to primarily associate with ergosterol in the fungal cell membrane and additionally believed to associate together to form a porelike scaffold causing loss of intracellular contents.[36] Surfaces used in this study are essentially a low-roughness two-dimensional conjugated polymer coating. It is likely that the short linkers between the polymer coating and polyene drugs prevent significant penetration through the fungal cell wall, thereby preventing association with ergosterol targets in the fungal cell membrane. Additionally, these compounds may not have sufficient conformational flexibility to associate into porelike scaffolds.

Nonergosterol specific mechanisms for the antifungal effects of polyenes (AmB) have also been suggested. Ionic and oxidative effects[16] may occur, since auto-oxidation of AmB may induce the production of free radicals that could damage cells.[41] However, we observed no antifungal activity for surface-conjugated polyenes that could be attributed to this mechanism.

This study sheds light on the role of nonspecific adsorption of antimicrobials to surfaces and ensuing the possible misinterpretation of the mechanism of action. Besides serving as a cautionary tale for similar research, this is an important example of a "negative result" which actually strengthens our general knowledge of fungal pathogens, how they colonize materials, and accessibility of drug targets. One outcome has been the establishment of a standard washing protocol applicable to our plasma polymer surfaces for confirming covalent surface attachment. In other published studies, we have fabricated antifungal surface coatings using a different drug class of antifungal drugs (echinocandins) and conjugated these to plasma polymer coatings in a similar manner.[8] In contrast, microbiological assays showed that these covalently bound drug surfaces maintained a strong antifungal surface effect even after the same stringent washing. This comparison was illustrated in Fig. 6 showing surfaces prepared with the echinocandin drug caspofungin (caspo) producing a >log 6 reduction in colony forming units. So effective is this antifungal surface coating that we use it as our positive control sample. The important difference between polyene- and echinocandin-conjugated surface coatings is that the target of the surface-attached compounds in the latter is the structural integrity of the fungal cell wall. While the biomechanics of how tethered drugs meet cellular targets in the envelope is still under investigation, comparative results with these two drug classes importantly reveal which targets of the fungal cell envelope may be accessible to drugs grafted onto biomaterials surfaces. This new knowledge would have been completely obfuscated had we been tempted to misinterpret the mechanism of action on the basis of washing results. A detailed

understanding of how pathogens adhere to surfaces, and which cellular structures are accessible to surface-conjugated chemicals, will become invaluable knowledge when developing advanced materials and coatings for preventing implant-related infections.

## IV. SUMMARY AND CONCLUSIONS

We have prepared an antifungal surface coating by conjugating polyene-class antifungal agents onto surfaces. Two populations were found to be present: an irreversibly attached population held by specific chemical bonds and a reversibly bound population formed by weak, nonspecific forces. After rigorous and prolonged washing using water, and buffer solutions, polyene-coated samples exhibited contact-killing properties, causing up to a log 6 reduction in viable fungal colonies. However, a more thorough analysis showed that when the reversibly adsorbed population was completely removed with surfactant washing at elevated temperatures, the antimicrobial proprieties of the polyene-grafted surfaces were eliminated. Thus, we conclude that the apparent antifungal properties of such coatings can be attributed to the diffusive release of compounds from the surface, whereas covalently attached polyenes were unable to prevent fungal attachment. This study importantly illustrates the possibility that an incorrect mechanism of action for antimicrobial surface coatings could be assigned even when rigorous aqueous washing or soaking protocols are assumed to have removed physisorbed compounds.

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